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The Effects of Different Size Gold Nanoparticles on Mechanical Properties of Vascular Smooth Muscle Cells Under Mechanical Stretching

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ABSTRACT

The field of nanotechnology research has seen a large growth in the past few decades due to the great potential of novel nano-size material for useful applications such as drug delivery and medical imaging. Also, they are a promising platform for the detection of terrible diseases such as cancer and atherosclerosis. Although these materials hold great promise, there is very limited understanding about the biological effects of these nanoparticles on normal healthy cells, such as vascular smooth muscle cells (VSMCs). The VSMCs are unique cells due to the two distinct phenotype characteristics it can express: a contractile and a synthetic phenotype. Normally, they express a contractile phenotype for their primary function of regulating the lumen diameter of blood vessels. They also can express a synthetic phenotype when vascular injury occurs.

In this study, the effects of different size citrate capped gold nanoparticles on the mechanical properties of vascular smooth muscle cells subject to mechanical stretching was investigated. The first part of the experiment assessed the cytotoxicity of the nanoparticle treatment on the cell. Next, the assessment of the mechanical properties was examined using atomic force microscopy (AFM) for cyto-indentation. The final part of the investigation was immunofluorescence staining to look at the alpha actin within the cell for possible phenotypic shift.

The study showed the citrate nanoparticles did not have any toxic effects on the cells, but the nanoparticle treatment did alter the mechanical properties and phenotypic expression of the VSMCs. Under static condition, the nanoparticle treatment decreased

the mechanical properties of the cells. The size of the nanoparticle also had a decreasing elastic modulus effect on the cell. The VSMCs subjected to mechanical stretching exhibited higher elastic modulus compared to the static experimental groups. The nanoparticle treatment also decreased the mechanical properties of the cells. However, the size of the nanoparticle did not have any influence on the decrease of the cells elastic modulus unlike the static treated cells. The mechanical testing condition provided a better look at how these particles would affect the cells *in vivo*. While the nanoparticles are not cytotoxic to the VSMCs, they are altering the mechanical properties and phenotype of the cell.

DEDICATION

I would like to dedicate this small piece of my life to my family and friends. I have put a lot of time and effort into completing this thesis, but nothing will compare to the time and effort my family and friends have spent supporting me, encouraging me, listening to me, making me smile every day of my life, and most importantly loving me no matter what.

I would especially like to thank my mom, dad, and little sister. The past year and a half has been really tough, and I do not know if I would have been able to accomplish everything I have done without you being there every step of the way.

"Con cảm ơn bạ và mẹ"

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Additionally, I would like to thank some of the graduate students in Multiscale Bioelectromechanics Lab (MBEM). I would like to thank Alex Lindburg for his guidance in the begin, Ruikai Chen and Aesha Desai for training and support using the AFM machine, and Cassie Gregory and Elliot Mappus for their assistance with cytotoxicity assays and staining protocols. I would also like to thank Jayesh Betala and Varun Chawla in Dr. LaBerge's lab for providing me with the cells and training me on the FlexCell System. Finally, I would like to thank Scott Cole and Ashley Hart, in Dr. Kitchens' lab, for providing me with the nanoparticles.

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CHAPTER ONE

INTRODUCTION

1.1 Motivation:

Nanotechnology research, such as development of different nanoparticles for therapy and imaging of different diseases have shown many promises, especially with possible detection and treatment methods for cancer. Within the past decade, an increasing number of research studies and publications using nanomaterials to treat and detect cardiovascular disease, such as atherosclerosis, are emerging. As with any disease, early detection will help aid the process of treatment and prevention of further development of the disease, but there is still limited understanding of the total effects of these nanoparticles on certain cells within our body.

The cardiovascular system is a significant area of research for many scientific, medical, or engineering disciplines. The cardiovascular system includes the heart and blood vessels that pump and transport different nutrients and waste from one part of the body to another. If the cardiovascular system becomes damaged, the entire body is affected. Cardiovascular disease is the leading cause of death in the United States. Many people in the U.S. do not recognize or realize the symptoms and indicators of cardiovascular disease; therefore, a lot of cases go undiagnosed until severe treatment is needed. Many cardiovascular diseases affect blood vessels, the major transportation pathway for blood through the body. In particular, atherosclerosis is a particular disease that occurs when cholesterol and triglycerides accumulate on the walls of blood vessels. Initially, the build-up has limited blood flow effects; however, once the disease

progresses, the accumulation begins to seriously constrict the fluid flow, a cascade of symptoms that can ultimately cause death occurs. Nano-technological research is emerging to tackle the challenges of atherosclerosis and the effects it has on the body. However, there is limited understanding about how these particles interact and affect normal cells, such as vascular smooth muscle cells. This lack of information could be dangerous if these nanoparticle interactions yield an adverse response. Therefore, an in-depth investigation is required to figure out the interactions between healthy cells and these nanoparticle with these nanoparticles, and how these interactions affect the overall characteristics, mechanical properties, and/or functions of the cells.

The purpose of this research is to improve upon the understanding of how these new nanomaterials, particularly gold nanoparticles, will affect the vascular smooth muscle cells found in blood vessels.

1.2 Research Goal:

Investigate the influence of different size citrate capped gold nanoparticles on the mechanical properties of vascular smooth muscle cells subjected to mechanical stretching.

Aim 1: Evaluate the cytotoxicity of gold nanoparticles on VSMCs

Aim 2: Evaluate the effects of the size of the nanoparticles on the mechanical properties VSMCs

Aim 3: Evaluate how the nanoparticles influence the phenotype expression by assessment of the shape, size, and alpha-actin distribution and arrange of the cell.

CHAPTER TWO

GOLD NANOPARTICLES IN BIOMEDICAL RESEARCH

2.1 Introduction:

In recent decades, the use of nanoparticles, particularly metallic nanoparticles, has increased in biomedical research.[1] The small size allows for interaction with DNA, proteins, lipids, and other cellular components. Common metallic nanoparticles studied include gold, silver, titanium oxide, and iron oxide nanoparticles.[2], [3] Gold nanoparticles (AuNPs) possess distinct chemical and physical properties and are one of the most widely investigated materials. [3-4] Gold nanoparticles unique properties include: (a) capable of synthesizing in a wide variety of sizes from 1 nm -100 nm, (b) controlled synthesis into various shapes such as nanospheres, nanorods, nanocubes, nano shells, etc., (c) large surface area to volume ratio, (d) stability over a wide range of temperatures, (e) easily functionalized as seen in **Figure 2.1** with different conjugates such as thiols, amines, DNA, enzymes, antibodies, and some functional polymers.[2] All these properties can be readily manipulated depending on the desired application. Thus, nanoparticles and the field of nanomedicine provide a promising platform for various solutions for the enhancement of different bioimaging modalities, drug delivery applications, cancer diagnosis and treatment, and many other biomedical applications. [4], [6]–[8] However, while nanotechnologies have shown great promise in these areas, long-term biological effects of the nanoparticle and cellular interaction are not fully understood. Some studies have shown adverse effects due to exposure of certain nanoparticles.[9], [10] Therefore,

there is a need for the investigation of the effects of these gold nanoparticles on cellular functions. Understanding the interaction between the cell and the gold nanoparticle is important for safe and effective use of these nanomaterials. In this review, biomedical research using nanoparticles, particularly gold nanoparticles, for drug delivery, imaging enhancement, cancer research, and cytotoxic studies will be explored.

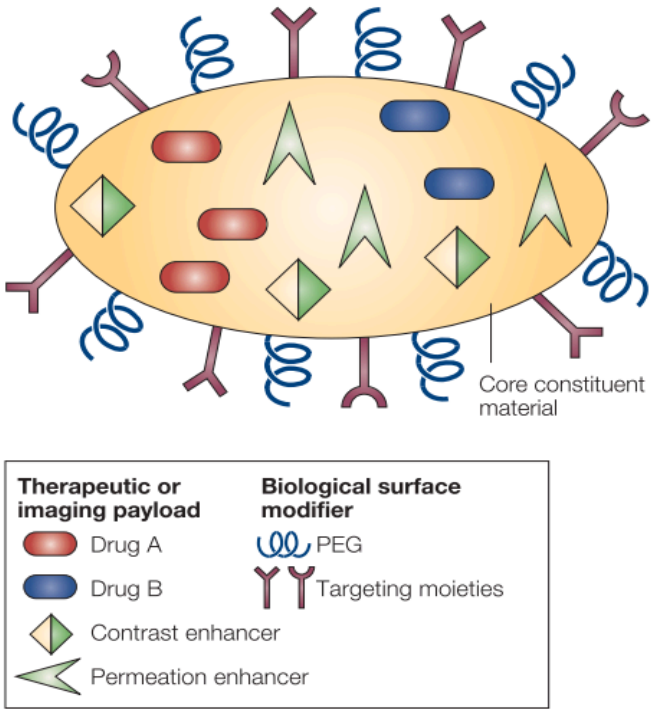


Figure 2.1: Multifunctional Gold Nanoparticle. The following are illustrated: the ability to carry one or more therapeutic agents; biomolecular targeting through one or more conjugates antibodies or other recognition agents; imaging signal amplication, and biobarrier avoidance by conjugated with polyethylene glycol (PEG) for avoidance of macrophage uptake.[6]

2.2 Gold Nanoparticles in Drug Delivery Applications

Targeted drug delivery system (TDDS) is among the most important research area in biomedical engineering and pharmacology. The goal of TDDS is to increase the concentration of the drug in the tissue of interest to enhance the therapeutic effects while leaving the normal, healthy tissue unaffected. The reasons nanoparticles are being researched for drug delivery applications are due to the small size, cellular interaction compatibility, large surface to volume ratio, and ease of functionalization of the particles.[2], [11], [12] These unique characteristics of nanoparticles allows large surface coverage of a desired drug on particles, via ligands or other biomolecules, that because of its size, will have the capability to bypass many bio-barriers in the body, target a particular tissue of interest, and reduce overall systemic toxicity. The incorporation of drugs onto the nanoparticle would help improve drug solubility; thereby, offering a more regulated drug release with enhanced retention at the target site.[13] One research group lead by Niidome has examined one of these challenges. To bypass the reticuloendothelial system (RES), which consists of cells that filter out and destroy bacteria, viruses, and other foreign substances, AuNPs were conjugated with thiol-derivative polyethylene glycol (PEG-SH). The study found that this PEG-SH outer coating on the cetyl trimethylammonium bromide (CTAB) capped AuNPs could delay RES clearance to the liver from 30 minutes to 72 hours in mice models, an approximately 150-fold improvement compared to unmodified CTAB capped AuNPs.[14] This controlled delay is a significant advancement for drug delivery applications. The unique properties nanoparticles offer enable scientists to research and solve more obstacles, such as how to

treat diseases in the brain, which is a significant challenge for many drugs cross the blood-brain barrier.[15]

Gold nanoparticle drug delivery has potential use as a feasible platform for the targeting and treatment of many cancers. Kudgus et al. have shown the utilization of Gemcitabine conjugated AuNPs inhibited the growth of Pancreatic Adenocarcinoma.[16] Another study successfully delivered Doxorubicin, anti-cancer drug, into the nucleus of melanoma cells in mice.[17] Many other drugs, such as Oxaliplatin, Paclitaxel, and Cisplatin, conjugated with AuNPs are being researched for different treatments of cancer such as melanoma and prostrate cancer.[1], [18]–[20]

Another drug delivery application with AuNPs includes loading them with biomolecules. Joshi et al. investigated the utilization of oral and intranasal administration of colloidal gold nanoparticles conjugated with insulin to diabetic rats in order to reduce blood sugar levels. The studied reported a significant decrease in blood sugar which was comparable with the decrease obtained by subcutaneous insulin injections.[21] Another study investigated the potential of AuNPs for vaccine delivery for the hepatitis B virus. These Chitosan-6-AuNPs have been studied for their efficiency concerning *in vitro* and *in vivo* applications. When delivered intramuscularly to mice, these conjugated AuNPs were more efficient than the naked DNA vaccine. The study also found these particles induced cytotoxic T lymphocyte responses at a lower dosage when compared to bare DNA.[22]

There exist numerous completed and on-going research studies on using nanoparticles as a drug delivery vehicle for the treatment of many diseases, such as

cancer. AuNPs offer many unique attributes allowing for novel drug delivery strategies to be discovered. The key issue is the engineering of these particles for targeting the specific site of interest, optimizing the bioavailability, and reducing any adverse response.

2.3 Gold Nanoparticles in Imaging Enhancement Applications

There are many imaging modalities such as X-rays, Computed Tomography (CT), Positron Emission Tomography (PET), and Magnetic Resonance Imaging (MRI) used daily in clinical settings. These analytical machines help medical professionals view the internal anatomy of a patient to look for abnormalities. Although these imaging methods are beneficial tools, there are still complications and limitations with each of the techniques. For example, iodine and bismuth are used as contrasting agents to address contrast resolution problems; however, these contrasting agents have limited circulation life due to fast filtration by the kidneys, toxicity of the agents at certain concentrations, and the limited control for agent site targeting. In the past decade, research regarding AuNP conjugates as a possible contrast agent for different imaging modalities has been explored.

One of the most widely used and inexpensive imaging techniques is the X-ray. X-rays provide rapid clear results of the interior of a patient's body. Because of X-ray low radiation doses, they do not provide the high soft tissue contrast that the CT and MRI scans offer. In order to increase x-ray contrast, some studies have explored using AuNPs. One study has effectively shown AuNPs were efficient enough to be used as an X-ray contrast agent *in vivo*. Hainfeld et. al. injected 1.9 nm AuNPs into mice. The injected

mice were x-rayed and blood vessels as fine as 100 μm in diameter could be distinguished. The animal study demonstrated AuNPs are useful x-ray contrasting agents that offer novel physical and pharmacokinetic advantages over current standard iodine based agents.[23]

Gold nanoparticles were also explored for their applications in improving CT and MRI imaging techniques. Kim et. al. performed an *in vivo* study using Sprague-Dawleys rats. The 20-50 nm diameter PEG coated AuNPs intravenously administered to the rats. The CT imaging results of the AuNPs treatment were compared to the current iodine-based contrast agent, Ultravist. The study found a greater efficiency in the contrast using the AuNPs compared to the Ultravist contrast agent. The AuNPs also had a improved circulation time and no renal toxicity; two limitations of using an iodine based contrast agent.[24] Another studied used Gadolinium Chelate coated AuNPs as a possible contrast agent for both CT and MRI scans. They also found that gold nanoparticles was a better contrast agent than currently used agents and no undesirable effects were observed during the administration of the nanoparticles or 6 weeks following the treatment.[25]

The use of AuNPs as a contrast agent in different imaging modalities is a feasible possibility for future clinical settings. However, before AuNP implementation in clinical settings, a lot more research has to be performed to study the effects the particles have on the human body. Much of the current investigations focused on the feasibility of AuNPs as a contrast agent and the possibility of using the particles as an imaging agent for the detection of cancer.[5], [26]–[29] There are also recent studies emerging that involve the use of nanoparticles for imaging cardiovascular diseases.[30], [31] These two focuses of AuNPs

will be discussed in more detail in the following sections. Overall, AuNPs have demonstrated their promising use in another biomedical research area.

2.4 Gold Nanoparticles in Cancer Research

Cancer is the third leading cause of death (after heart disease and stroke) in developed countries and the second leading cause of death (after heart disease) in the United States.[27] According to cancer statistics performed by the American Cancer Society in 2010, near 600,000 deaths per year are a direct result from cancer and almost 1.5 million new cancer cases are diagnosed yearly in the U.S. alone.[32] The highest survival chance for patients diagnosed with cancer relies on early detection and prevention methods. The unique properties of AuNPs offer various advantages that are currently being studied to assist with cancer detection, treatment, and prevention.

There are many different studies investigating the effects of AuNPs on different types of cancer, see **Table 2.1.** These experiments all demonstrate the plausible applications of AuNPs for cancer diagnosis and treatment. For instance, Wang et. al. used acetylated dendrimer-entrapped gold nanoparticles (ADENPs-AuNPs) for the imaging of human lung adencarinoma cell lines *in vitro* and *in vivo*. The results showed the ADENs-AuNPs were able to assist in the detection of the prescence of cancer cells using CT scans. The study also indicated no sign of cytotoxicity due to the AuNPs at the concentration used in the study.[29]

Besides just detecting the presence of cancer cells, researchers are hoping to use AuNPs as a therapeutic agent to treat cancer tumors. Researchers at the Mayo Clinic looked at the effects of Gemcitabine conjugated AuNPs on the growth of pancreatic

adenocarcinoma *in vitro* and *in vivo*. The study found that these particles were effective in inhibiting the growth of the tumor in advance stage models. Further research is being conducted to improve the efficacy of the nanosystem and gain greater understanding about the pharmacokinetics.[16] However, not all the studies have shown promising results. One studied looked at 5nm diameter Gadolinium conjugated AuNPs effects on MC7 breast cancer cells. The *in vitro* study showed promising results with a cytotoxic effect of up to 55% on the cancer cells. However, the *in vivo* studied showed no significant difference between the treated and untreated mice.[19]

More work still needs to be completed before AuNPs could be a robust and highly effective option for the treatment of cancer in the clinical setting. The field of nanotechnology has made a significant amount of progress, but there are still many unanswered questions regarding the use of AuNPs for the treatment of cancer. There is huge potential for the use nanoparticles in cancer therapy, and with the intense global interest in nanotechnology and nanomedicine; it is likely that many of these questions will be addressed in the future.

*Au*NPs: size and functionalization	Conjugated/Targeted	Type of cancer	Techniques/Methods	Advantages
25 nm	anti-EGFR	Precervical cancer	Illumination microscopy, combined with novel contrast agents	Powerful tool for detecting cellular and molecular changes
PEG-modified *Au*NPs	Herceptin (HER)	Breast cancer cells	Molecular imaging	Promising imaging technique for early detection of cancer
15 nm	anti-EGFR	Oral cancer	SERS	Saliva-based assay for early diagnosis of oral cancer
60–80 nm pegylated	ScFv (single-chain variable fragment)	Head-and-neck	SERS	Optical and EM probes for tumor detection
Au nanorods	anti-EGFR	Oral squamous cell carcinoma	SERS	Bioimaging and cancer biodiagnostics
45 nm ∗ 15 nm *Au* nanorod	Her-2/neu antigen	Prostate cancer detection	Contrast photoacoustic imaging	Visual tool for molecular and structural information
15 nm	F19 monoclonal antibodies	Pancreatic adenocarcinoma	Light scattering, size exclusion chromatography, and TEM	Novel labeling method
20 nm	Aptamer	Leukemia and lymphoma	Spectroscopic method	Early and accurate detection of cancer
Oval shaped (14 nm length and 18 nm width)	anti-HER2/c erb-2 antibody and aptamers	Breast cancer	Colorimetric and two-photon scattering	Rapid and highly sensitive
*Au*NPs (size not known)	Tag-PC-3	Prostate cancer	Photoacoustic waves	Detect single cells under flow condition
Au functionalized with PEG (90 nm)	EGF-ligand and tag Raman receptor	Circulation tumor	SERS	New clinical tool for management of patients with SCCHN
15 ± 2 nm	anti-CA15-3-HRP antibody	Breast cancer	ELISA	Cancer biomarker detection
20 nm	cationic protein kinase C (PKC-alpha) peptide	Breast cancer	GNP-based colorimetric assay	Initial screening during cancer diagnosis
100 to 150 nm	anticarcinoembryonic antibody	Cancer	ELISA	Early diagnosis of cancer
30 nm (±10)–150.5 (±30.4) nm	ENO1 antibody	Lung cancer	Electrochemical-based immune sensor	Quantitative tests protein and cancer biomarkers
15 nm	Functionalized glassy carbon electrode (GCE)	Lung and liver cancer, drug-sensitive leukemia cells, and drug-resistant leukemia	Electrochemical and contact angle measurements	Rapid identification and highly sensitive detection for cancer

Table 2.1: This table list the research on using gold nanoparticles for the possible detection and treatment of different type of cancers.[32]

2.5 Cytotoxicity of Gold Nanoparticles

Gold nanoparticles are intentionally engineered to interact with cells; therefore, it is important to ensure that these modifications do not cause any adverse effects in the body. For biomedical purposes, especially *in vivo* applications, toxicity is a main factor to investigate when evaluating the potential and effectiveness of the nanomaterial. Cytotoxicity is one of the most common criteria used to assess the effects of the nanoparticles on cells and the particles viability for use within the body. However, studies analyzing toxicities of AuNPs have a significant amount of uncertainty and are difficult to compare quantitatively. The difficulty occurs because of variability in the type of nanoparticles, different size particles, different cell lines, concentration, exposure time, incubation time, and cytotoxicity assays used for the analyses.[3] Despite these variables, there are trends that can be extracted and compared qualitatively from the many studies.

One of the parameters many investigations have examined is the effect of size on the toxicity of the cell. A particular study performed by Coradeghini et.al. investigated mouse fibroblasts (3T3s) exposed to citrate stabilized AuNPs.[33] The authors presented evidence that 5nm diameter citrate capped AuNPs were cytotoxic to the 3T3s at concentrations greater than 50 μM. Meanwhile, 3T3s exposed to 50, 100, 200, and 300 μM of 15nm diameter citrate stabilized AuNPs had no indication of toxicity.[33] Another study performed by Pernodet et. al. reported that 14nm diameter citrate capped AuNPs decreased cell proliferation and possessed slight signs of toxicity in human dermal fibroblasts.[9] The toxic effects observed in the study were attributed to actin stress.

However, a second, independent study performed by Li et.al. also reported the same effects in the presence of citrate-capped AuNPs with human lung fibroblasts, but the presented evidence actually indicated that cytotoxicity was a result of oxidative damage.[34] Connor et al researched utilizing human leukemia cells (K562) with particle sizes ranging from 4 to 18 nm in diameter and surface modifiers of citrate, biotin, and cysteine, and glucose. These cells showed no signs of toxicity at concentrations up to 250 μM.[3],[35]

Another study by Liu et. al. compared the effect of surface charge of AuNPs and the correlation it had with the cytotoxicity level. The results revealed that when using a MTT Cytotoxicity assay on HepG2 cells, positively charged 10-(mercaptodecyl)-trimethyl-ammunium bromide (AuNP-TMA) exhibited higher cytotoxicity than the negatively charged 11-mercaptoundecaonic acid (AuNP-MUA). Conversely, it was determined that negatively charged AuNP-MUA showed a higher cytotoxicity level than the positively charged AuNP-TMA in RAW 264.7 cells when using the same MTT assay. Utilizing a LDH assay indicated that a positively charged AuNP-TMA had a higher toxicity effect than the negatively charged AuNP-MUA in not only the HepG2 cells, but also the RAW 264.7 cells.[36] The authors found in this study that toxicity did not show significant physiochemical property, such as size and surface functionality, dependence. Rather, this study indicated that cytotoxicity was more reliant on cell interactions with AuNPs between the two different cell lines and the type of colorimetric assay used to assess the cell viability.[36]

There exist many other cytotoxic studies examining the effects of size, shape, surface chemistry, uptake, concentration, colorimetric assayss, cell types, and culturing conditions.[3], [37]–[41] Based on these current findings, the amount of cytotoxicity derived from the AuNPs versus other experimental variables and methods is unclear. Thus, conflicting data exist with regard to the toxicity of AuNPs that requires further examination to fully understand the possible adverse effects from AuNPs.

2.6 Conclusion

In summary, AuNPs have emerged as a promising alternative or additive for many different biomedical applications, such as drug delivery, medical imaging, and cancer diagnosis and treatment. Nanotechnology could also be a useful tool for other diseases such as Tuberculosis and Alzheimer's. Further research to fully understand AuNPs and their effects within the human body, must be performed before clinical trials can occur. Nevertheless, nanotechnology and its potential applications are expected to provide significant advancements in medicine.

CHAPTER THREE

THE SIGNIFICANCE OF VASCULAR SMOOTH MUSCLE CELLS

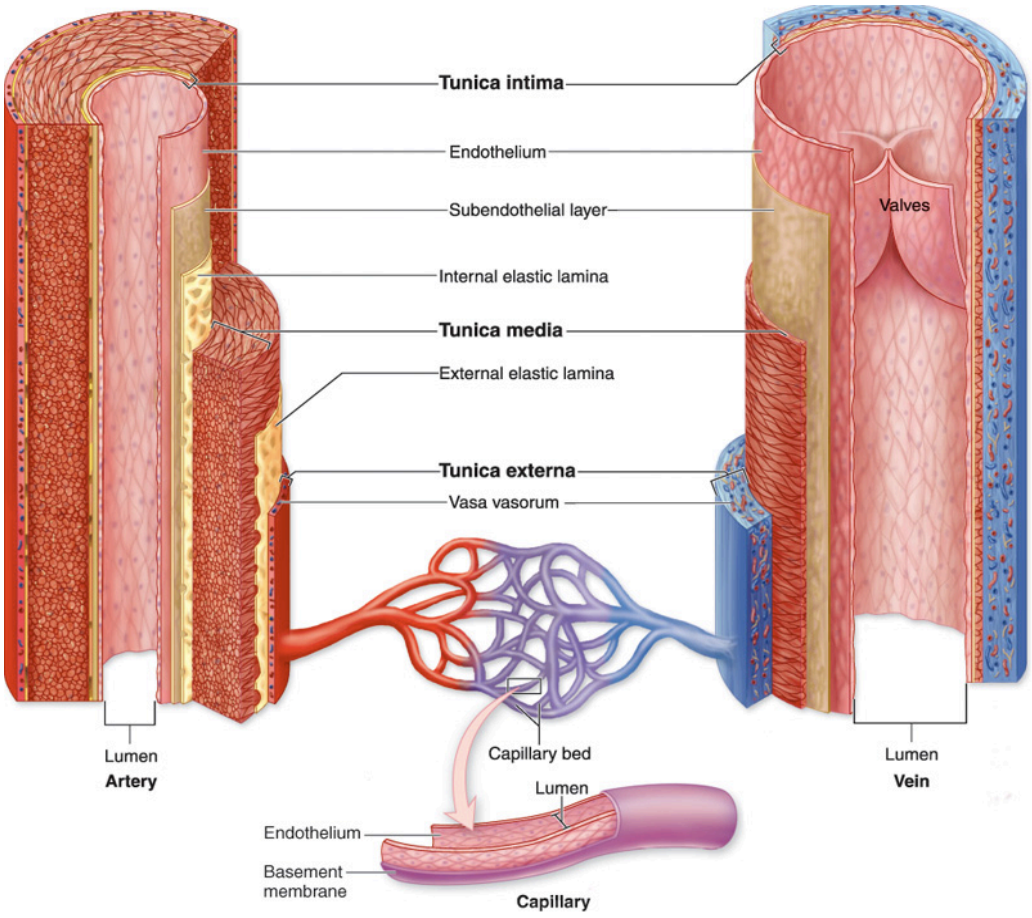


Figure 3.1: The anatomical breakdown of the different layers of arteries, veins, and capillaries. Blood vessels are made up of three different layers: tunica intima, tunica media, and tunica externa. Vascular smooth muscle cells (VSMCs) make up most of the tunica media in arteries and veins.[42]

3.1 Background of Vascular Smooth Muscle Cells

The human body contains a vast network of blood vessels that delivers nutrients and oxygen throughout the body. The vessels can be categorized into three different types of structures: arteries, veins, and capillaries.[42] Arteries and veins are constructed out of layers.[42] The innermost layer is the tunica intima, which is the main layer that is normally in contact with the blood.[42] The tunica intima is composed of simple squamous epithelial cells (endothelium lining) and a sub-endothelial layer of connective tissue. The endothelium lining creates a smooth surface blood to flow without damage.[43] The middle layer of the blood vessel is called the tunica media. The tunica media is comprised mostly of circularly arranged vascular smooth muscle cells (VSMCs), but other material such as collagen (type I, III, and V), elastin, and proteoglycans are also found in this layer. The tunica media's main function is to contract and relax the blood vessel walls to maintain steady blood flow. The outermost layer is the tunica externa or adventitia. This layer is comprised mostly of loose connective tissue made up of type I collagen fibers and some elastin fibers. This outer layer assists in anchoring the vessels within the body and counteracts external forces, such as longitudinal stretching.[44] All three layers shown in **Figure 3.1** make up the arteries and veins in the cardiovascular system throughout the human body.

Vascular smooth muscle cells are specialized cells designated to regulating the lumen diameter in blood vessels. This diameter control dictates contraction and dilation of the vessel walls and determines the blood distribution and pressure throughout the

body. Another special characteristics of VSMCs are that they are able to change their phenotype contractile or synthetic phenotype, between contractile or synthetic, in response to environmental factors. (see **Figure 3.2**)

Normally, VSMCs in the tunica media express the contractile phenotype. A significantly low rate of proliferation is exhibited and the cells express specific and unique contractile proteins.[45] The other phenotype VSMCs can undergo change into is the synthetic or proliferative phenotype. The synthetic phenotype expression is characterized by high proliferation rate, migration, and extra cellular matrix secretion, especially collagen type III and fibronectin. This phenotype is typically expressed by the VSMCs in response to vascular injury and plays a vital role in vascular repair. However, the downside to this phenotypic modulation or switching prompts the cells to respond to environmental signals readily leading to development of vascular diseases such as atherosclerosis, hypertension, and neointima formation. [34]

3.2 Research Interest in Vascular Smooth Muscle Cells

The ability of VSMCs to change their phenotype based on environmental stimulus plays a critical role in vascular repair. Unfortunately, the altering phenotype characteristic is also a main factor in the development and progression of atherosclerosis. Researchers are beginning to better comprehend some of the molecular mechanisms and factors that controls transitions in the phenotypic state of the VSMCs, but little is known about what controls the etiology and advancement of the different stages of atherosclerosis. Atherosclerosis is considered a chronic and complex disease that can ultimately result in the occlusion of the affected arteries and cause life-threatening

damage such as myocardial infarction or stroke. This disease usually begins with some sort of endothelial dysfunction or damage, which allows for increase accumulation of plasma-derived lipids and their oxidation products into the matrix of the arterial wall. The intimal cells react by recruiting inflammatory cells to the site of injury, initiating the migration of monocytes into the vessel wall. The monocytes become macrophages and ingest the oxidized lipid; thus, forming what many research call "foam cells". The recruitment of macrophages, and the production of inflammatory cytokines, oxygen free radicals, and other cascade factors lead to a downstream effect. This chain of reactions induces the migration of the VSMCs from the medial layer into the intima layer. The VSMCs begin to proliferate and synthesize matrix molecule to remodel the vascular wall a process also known as neointimal hyperplasia. This process continues until the lumen of the blood vessels becomes increasingly occluded and eventually leads to a blockage of blood flow.[46], [47] A schematic of the process of the development of atherosclerosis can be seen in **Figure 3.3**.

Current treatment for atherosclerosis includes using grafts to bypass the area of blockage, or implanting a vascular stent to open up the occluded vessel. However, these solutions possess unique complications and side effects. There exist many current biomedical research projects focusing on the development of tissue-engineering blood vessels to try to combat this disease. Emerging nanotechnology research aims to use nanomaterials to detect early signs and possibly even treat atherosclerosis.

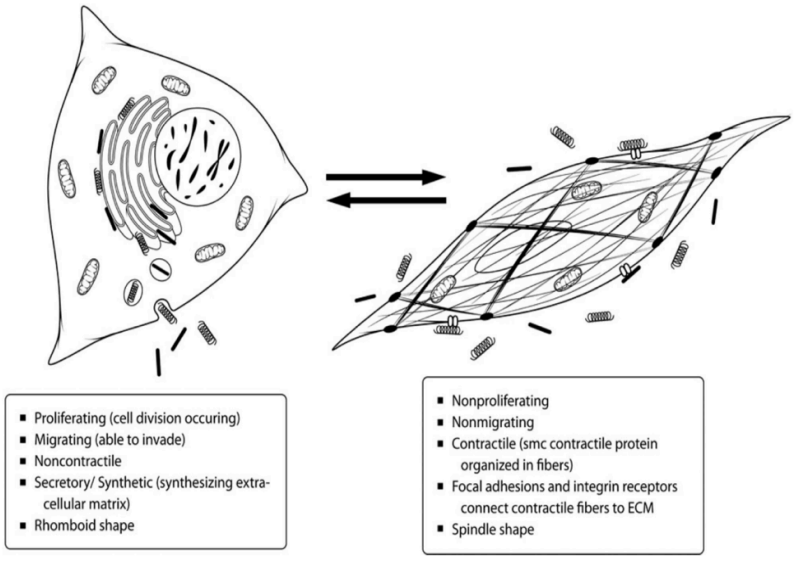


Figure 3.2: Phenotypic modulation or switching of vascular smooth muscle cells. The left side illustrates and list characteristic the characteristic of synthetic or proliferative phenotype. The main feature to focus on is the lack contractile fibers, increase organelles for the production and secretion of extracellular matrix. The right side illustrates and list characteristic the characteristic of contractile phenotype. The main feature to notice is the abundance of contractile fibers spanning the cell body. [46]

3.3 Conclusion:

Vascular smooth muscle cells are important because they help blood vessels in the body to distribute and maintain a steady blood flow, that provides nutrients throughout the body. VSMCs have the ability to change phenotype in response to different environmental stimuli such as mechanical or chemical factors to assist in maintaining a stable environment. These cells also are significant in the development and progression of atherosclerosis. Research with regards to VSMCs will further the understanding about the type of factors or even new materials such as nanoparticles, that will affect there phenotypic change and the ramifications these alterations will ultimately have on the body.

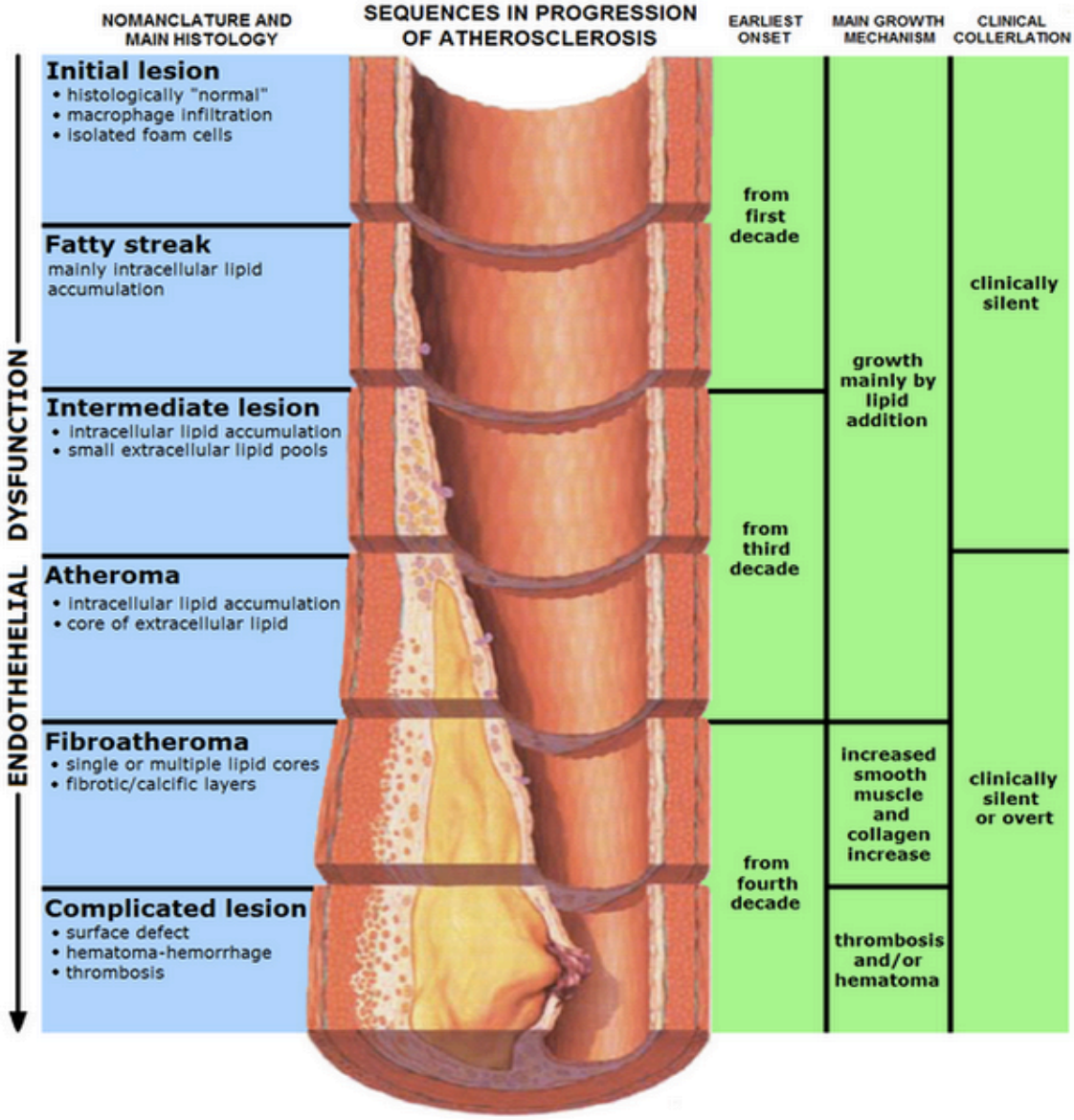


Figure 3.3: Development of Atherosclerosis. The disease begins with of damage to the endothelium lining or endothelial dysfunction. The image shows different stage of the disease and what each stage looks like. An important factor to notice is throughout the entire development and progression of atherosclerosis the disease is deemed clinically silent up until the advance stages.[42]

CHAPTER FOUR

THE EFFECTS OF DIFFERENT SIZE GOLD NANOPARTICLES ON THE MECHNICAL PROPERTIES OF VASCULAR SMOOTH MUSCLE CELLS UNDER PHYSIOLOGICAL STRESS

4.1 Abstract:

Nanotechnology is an emerging and promising frontier for medicine and biomedical research due to its potential for applications such as drug delivery, imaging enhancement, and cancer treatment. While these materials may possess significant possibilities, the effects of these particles in the body and how the particles affect the cells is not fully understood. In this study, vascular smooth muscle cells (VSMCs) will be exposed to 5 and 20 nm diameter citrate AuNPs under mechanical conditions. The cytotoxicity properties of these particles will be investigated using LDH and MTT assays. Atomic force microscopy will be used to study how the size of the nanoparticles affect the mechanical properties of the VSMCs. Immunofluorescence staining for alpha actin will also be performed to enhance understanding of the phenotypic shift. The LDH and MTT cytotoxicity assay results demonstrated that neither 5 nor 20 nm diameter nanoparticles are cytotoxic to the cells. However, the mechanical properties and cell morphology of the VSMCs was altered. Under static conditions, both AuNP treatments decreased the mechanical properties of the cells. The size of the nanoparticles had a softening effect on elastic modulus of the cell and sign of a synthetic phenotype was observed. The VSMCs subjected to mechanical stretching exhibited higher elastic modulus compared to the static experimental groups. Again, both AuNPs treatments

decreased the mechanical properties of the cells and signs of more synthetic phenotype was seen. However, the size of the nanoparticles did not have any influence on cell's elastic modulus unlike the static treated cells. The mechanical testing condition provided a better look at how these particles would affect the cells *in vivo*. While the nanoparticles are not cytotoxic to the VSMCs, they are altering the mechanical properties and phenotype of the cell.

4.2 Introduction:

In recent decades, the use of nanoparticles, particularly the use of AuNPs has increased in biomedical research. Their small size and unique properties allow these particles to interact with cellular components. Gold nanoparticle unique properties include: (a) wide variety of controllable sizes from 1 nm -100 nm, (b) Controlled shape variety, such as nanospheres and nanorods (c) large surface area to volume ratio, (d) functionalized readily with different conjugates.[2] All these properties can be engineered and altered depending on the particles intended application. Thus, AuNPs provide a potential platform for development and application for the future of modern medicine.[4], [6]–[8] However, while nanotechnology has shown great promises, some biological effects of the nanoparticle and cellular interaction are not well understood. Some studies have shown adverse effects on the body as results of nanoparticles exposure. Understanding the interaction between the cell and the AuNPs will further the overall understanding of how to use these nanoparticles properly and how they can be applied in different biomedical applications, such as medical imaging, drug delivery, or tissue engineering. Therefore, there is a need for the further investigation of the effects of these AuNPs on cellular functions.

Vascular smooth muscle cells (VSMCs) play an important role in contracting and relaxing the blood vessel walls to control blood flow and pressure. They also play a critical role in vascular repair because of their unique capability to change phenotype when vascular injury or other environmental factors occurs.[46]–[48] As the field of nanotechnology moves into studying cardiovascular diseases[31], the understanding for

how these nanoparticles affects cells in the cardiovascular system is substantially more important.

One of the most important effects to investigate is the cell toxicity level when exposed to these nanoparticles. Many nanoparticle studies have investigated the potential cytotoxic effects of nanoparticles on various cell types.[3], [41], [49] However, there is not a large understanding of the toxic effects these particles have on VSMCs. Common toxicity assays used include the Lactate Dehydrogenase (LDH) assay and MTT assay. The LDH assay is a colorimetric assay that measure cytotoxic level by measuring the plasma membrane integrity. Lactate Dehydrogenase, which is a soluble cytosolic enzyme present in the cell, releases upon cell death due to damage to the plasma membrane damage. The MTT assay measures cytotoxic levels by quantifying the cell metabolic activity based on the ability of mitochondrial succinate/tetrazolium reductase system to convert the yellow dye (MTT) to purple formazan.

Another interesting factor that will be examined in this study is how a dynamic *in vitro* testing condition will effect the mechanical properties of the cells, particularly VSMCs, compared to just a static experimental conditions. Overall, there exist few studies that actually evaluate how these nanoparticles influence the cells under mechanical conditions. The human body is not a static object. The human body contains chemical stimulation and mechanical stimulation. Investigating how these nanoparticle affects the VSMCs under mechanical conditions *in vitro* will help improve upon the understanding of the potential effects of these nanoparticle *in vivo*.

This study will evaluate how 5 nm and 20 nm diameter citrate capped AuNPs affect the mechanical properties of VSMCs under static and dynamic conditions, the cytotoxic effects on the cell, and phenotypic shift due to exposure to the nanoparticles.

4.2 Material & Methods:

Cell Culturing:

Aortic VSMCs were isolated from adult Sprague Dawley rats. The VSMCs were cultured in High Glucose Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) with 10 % fetal bovine serum (FBS) (Sigma) and 1% antibiotic-antimycotic solution (Sigma). VSMCs were incubated in polystyrene T-75 flasks at 37°C and 5% CO_2. The cell culture media was replaced every 48 hours. They were grown to approximately 70-80% confluence in flasks. Between passages, the cells were trypsinized with 0.25% trypsin with 0.02% ethyldiaminetetaacetic acid (Sigma). Cells between passage 3 and 8 were used for experiments.

Nanoparticles Synthesis:

The 5 nm diameter citrate capped spherical AuNPs were prepared by using100 μL of a 0.1 M citrate solution and 100 μL of 0.05 M of tetrachloroaurate (III) ($HAuCl_4$). The solution was next diluted in 20 mL of deionized water. The aqueous gold/citrate mixture was then reduced by the addition of 100 μL of 0.05 M of sodium borohydride ($NaBH_4$). This synthesis yielded a ruby red AuNP dispersion.

The 20 nm diameter citrate capped spherical AuNPs were prepared by adding 500 μL of 0.05 $HAuCl_4$ to 50 mL of deionized water. The solution was then heated.

Next, 1 mL of 0.1 M citrate solution was added. This solution was stirred and heated for at least 10 minutes. This synthesis method yielded a ruby red AuNP dispersion

Transmission Electron Microscopy (TEM) was performed to confirm the relative size of the nanoparticles.

Flex Cell System:

The VSMCs were seeded into 6-well collagen plates (BioFlex) with 25,000 cells per well. The cells were allowed to incubate over night to adhere to the plate. For the static conditions and dynamic, 3 groups were made: control cells (CC), 5nm citrate capped gold nanoparticle (5AuNPs), and 20nm citrate capped gold nanoparticles group (20AuNP). These groups were also made for the dynamic conditions. Once the cells were ready, 25 μM of phosphate buffered saline solution (PBS), Citrate solution, 5nm AuNPs, 20 nm AuNPs were added to the CC, CCS, 5 nm AuNP, and 20 nm AuNP group, respectively. To provide the dynamic condition, the Flex Cell 3000 System was used. The plates were loaded onto the platform and place inside the incubator. The plates were pre-conditioned under 0-4 % cyclic strain at 0.1Hz for 30 minutes and 0-4% cyclic strain at 0.5 Hz for 30 minutes. Finally, the groups were allowed to run for 72 hours under 0-4% cyclic strain at 1.0 Hz.

Cytotoxicity Assays: LDH

The LDH assay kit used was CytoTox96® Non-Radioactive Cytotoxicity Assay (REF#: G1781 & G1782) purchased from Promega Corporation. The VSMCs were seeded into a 96-well plate at 10,000 cells per well. The cells were allowed to incubate over night to adhere to the cell plate. The AuNPs were added at so that there were 5 μM,

25 μM, and 50 μM concentration in the experimental wells. Each sample group contained 6 wells. After treatment period of 3 days, the lysis solution was added at a ratio of 15 μL per 100 μL of media. Incubated the well with the lysis solution for 60 minutes at 37°C and 5% CO_2. Next, 50 μL of the supernatant of each of the wells were transferred to a new 96 well plate. Then 50 μL of the reconstituted Substrate Mix was added to each well. The plate was covered and incubated at room temperature for 30 minutes. Finally, 50 μL of the Stop Solution was added to each well and the absorbance reading was taken at 490 nm.

Cytotoxicity Assays: MTT

The MTT assay kit used was CytoTox96® Non-Radioactive Cell Proliferation Assay (REF#: G4001 & G4002) purchased from Promega Corporation. The VSMCs were seeded into a 96-well plate at 10,000 cells per well. The cells were allowed to incubate over night to adhere to the cell plate. The AuNPs concentration were added at 5 μM, 25 μM, and 50 μM to the experimental wells. Each sample group contained 6 wells. After a treatment period of 3 days,,15 μL of Dye Solution was added to each well. The plate was allow to incubate for 4 hours at 37°C and 5% CO_2. After the incubation period, the media/dye solution was removed and 100 μL of the Solubilization/Stop Solution was added to each well and the absorbance reading was taken at 570 nm.

Immunofluorescence Staining:

Immunofluorescence staining was performed to look at the smooth muscle alpha-actin to see if there might be a phenotype change has occurred in the VSMCs based on the alpha acting concentration and distribution. First, the cells were fixed in 4%

Paraformaldehyde for 10 minutes and then washed 3 times with PBS. Next, the cells were then permeabilized with a solution of PBS/0.01M Glycine/0.2% Triton-X (Sigma) for 30 minutes. Then, the cells were incubated in blocking solutions with 10% Goat Serum/PBS for 15 minutes. The cells were incubated with a dilution of 1:500 solution of Monoclonal Anti-Actin, Alpha-Smooth Muscle Antibody produced in Mice (Sigma Aldrich) with the blocking solution overnight at 4°C. After the cells were incubated over night, the cells were washed with PBS 3 times. Next, the cells were soaked in a dilution of 1:500 solution of Goat Anti-Mouse IgG(H+L) Fluorescein Conjugated Secondary Antibody (Millipore) with PBS for 2 hours protected from light. After washing again with PBS 3 times, the cells were next stained with a SlowFade Gold anti-fade reagent with DAPI (Invitrogen). The fluorescence images were taken using EVOS®FL Cell Imaging System (Life Technologies).

Atomic Force Microscopy (AFM):

Atomic Force Microscopy cytoindentation was performed on each sample 1- 24 hours after the 72 hours of static and dynamic (Flexcell stretching) conditions. The collagen membrane was carefully removed prior to testing and placed into a small AFM fluoro dish with 3 mL of new warm (37°C) media added into the dish. For the AFM testing, the Asylum Research MFP-3D was operated in contact mode with a fluid cell. A 5 um diameter borosilicate spherical-tipped AFM probed with a spring constant of 0.2 N/m was used to mechanically indent into each individual cells. The AFM optical microscope (40x) was used to position the tip of the cantilever over the cell center of the

cell. For each sample, every cell was indented 5 times to approximately 1 um depth to yield 5 force curves.

Force Curve Analysis of Data:

The force curves were exported from the AFM software and a series of MATLAB scripts was utilized to analyze the data. All MATLAB scripts used for the analyze in this experiment can be found in Appendix B. Next, a contact point was established from the dataset at a displacement value where there is a rapid increase in the deflection. This quick increase indicated contact between the cell and the tip. The offset for the deflection was adjusted in the MATLAB code. After the adjustment, MATLAB measures the cell stiffness/the apparent elastic modulus of the cell was calculated by fitting the data to the Hertz Model. The model was fitted to the first 100-200 nm of data. The Hertz Model used for a spherical indenter is defined in **Figure 4.1**.

$$F = \frac{4}{3}\frac{E}{(1-\nu^2)}R^{\frac{1}{2}}\delta^{\frac{3}{2}}$$

Figure 4.1 (Above): Hertz Model used for a spherical indenter. F and d represents the measured force and indentation depth respectively. The R represents the radius of the tip, which was set to 2.5 um. The ν represents the Poisson's Ratio, which was set to 0.5. The variable E is the elastic modulus and the variable of interest in this equation.

Statistical Analysis:

A Student's t-test was performed on the data to compare the control conditions to the experimental conditions to determine if any statistically significant differences existed between the samples. A P-value of less than 0.05 was considered statistically significant.

4.3 Results:

TEM of the Citrate Capped Gold Nanoparticles:

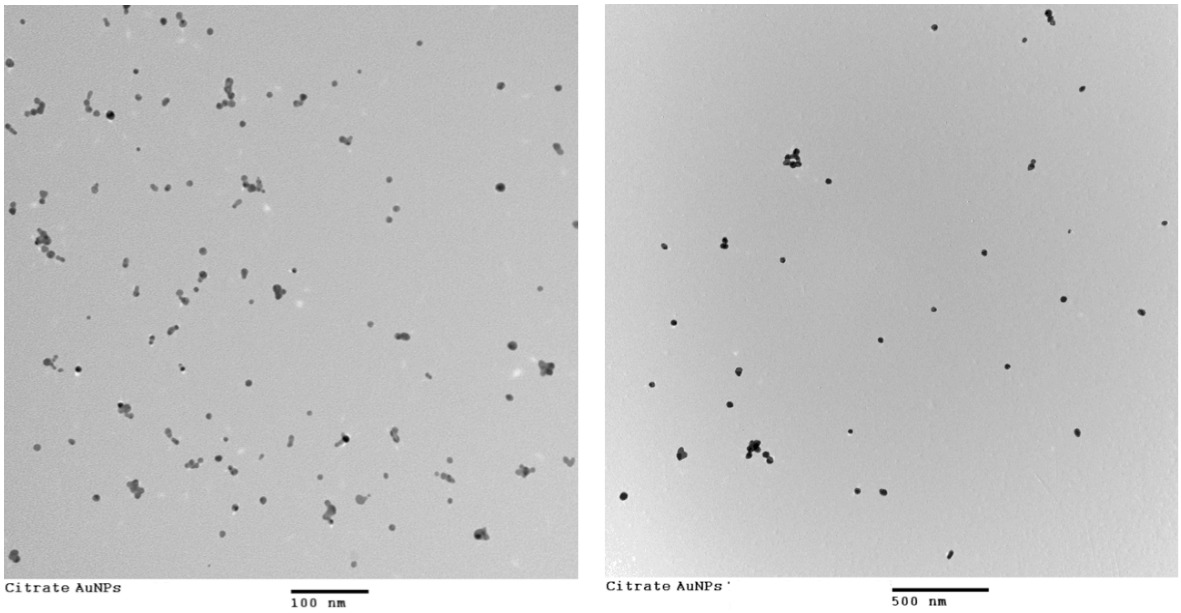


Figure 4.2: TEM image of the 5nm diameter (Left) and 20 nm diameter (Right) citrate capped AuNPs.

TEM was performed to confirm the relative size of the nanoparticle. The image on the left in **Figure 4.2** shows the 5 nm citrate capped AuNPs. The sizes of these particles are relatively around 5nm. The image on the right in **Figure 4.2** shows the 20 nm citrate capped AuNPs. The sizes of these particles are relatively around 20 nm.

Cytotoxicity Assays: LDH

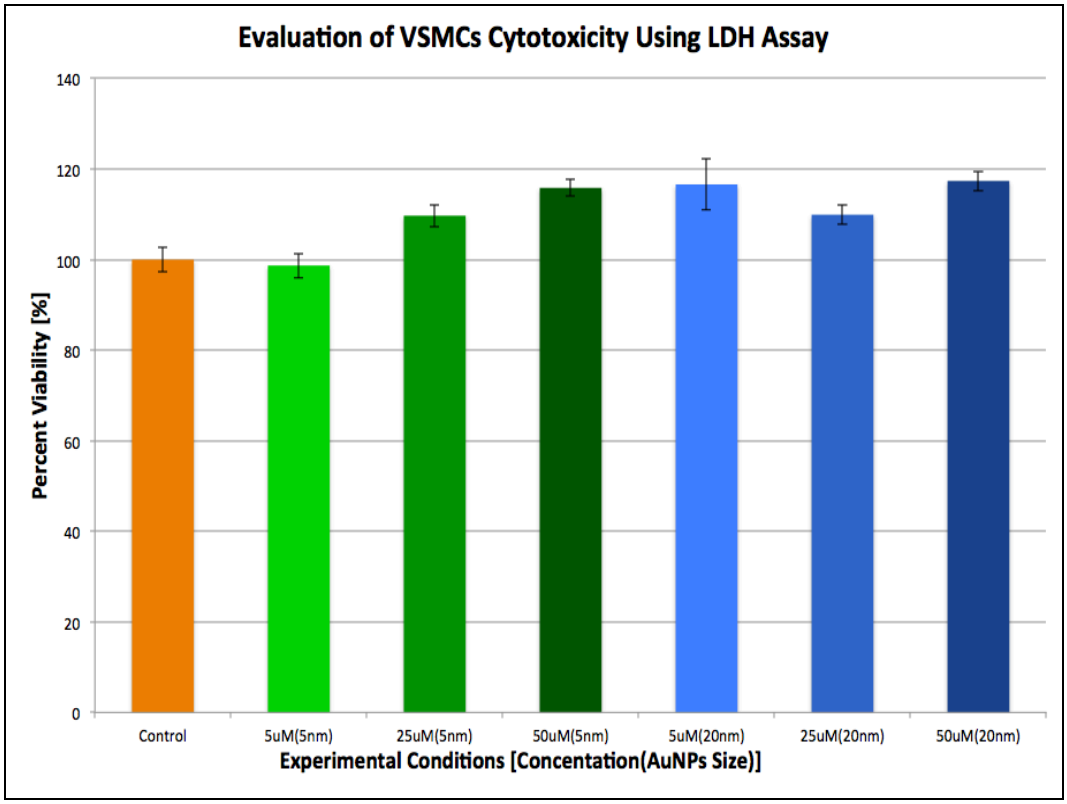


Figure 4.3: The results for the LDH Cytotoxicity on VSMCs treated with different concentrations of 5nm and 20nm citrate capped AuNPs.

The LDH Cytotoxicity results showed that VSMCs treated with 5 μM, 25 μM, and 50 μM of the 5nm AuNPs had a viability percentage of 98.68% ±2.72, 109.69% ±2.43, and 115.84% ±1.82, respectively. The 5 μM, 25 μM, and 50 μM of the 5nm AuNPs showed viability percentage of 116.60% ±5.61, 109.92% ±2.13, and 117.36% ±2.14. There were no adverse signs of cytotoxicity on the VSMCs under all the tested conditions. Some of the groups showed a higher viability percentage than the control.

Cytotoxicity Assays: MTT

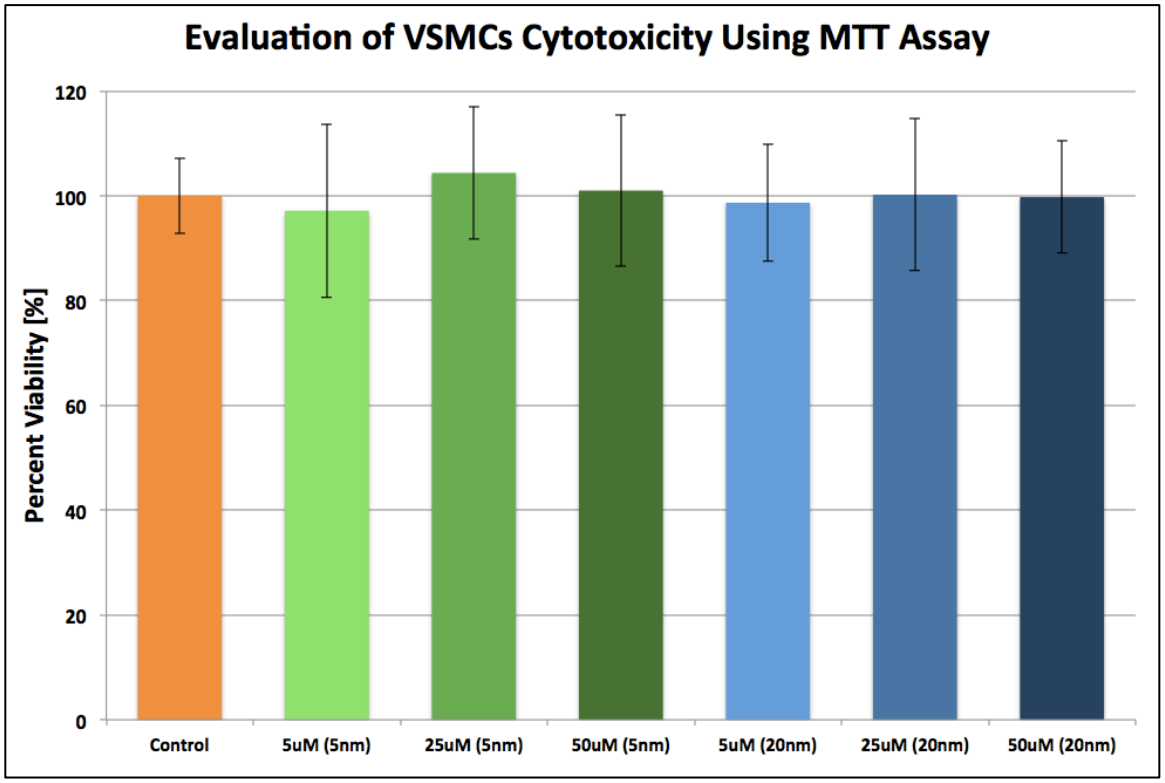


Figure 4.4: The results for the MTT Cytotoxicity on VSMCs treated with different concentrations of 5nm and 20nm citrate capped AuNPs.

The MTT Cytotoxicity results showed similar trends to the LDH Cytotoxicity results. Again, no apparent cytotoxic effects were observed. The VSMCs treated with 5 μM, 25 μM, and 50 μM of the 5nm AuNPs had a viability percentage of 97.16% ±15.77, 104.35% ±12.02, and 100.98% ±12.02, respectively. The 5 μM, 25 μM, and 50 μM of the 5nm AuNPs showed viability percentage of 98.67% ±11.13, 100.21% ±14.47, and 99.75% ±10.65. There were no adverse signs of cytotoxicity on the VSMCs under all the tested conditions.

Immunofluorescence Staining:

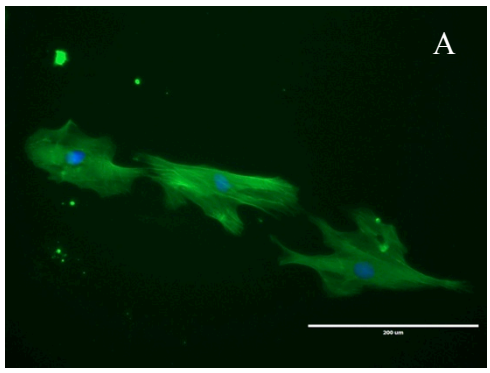
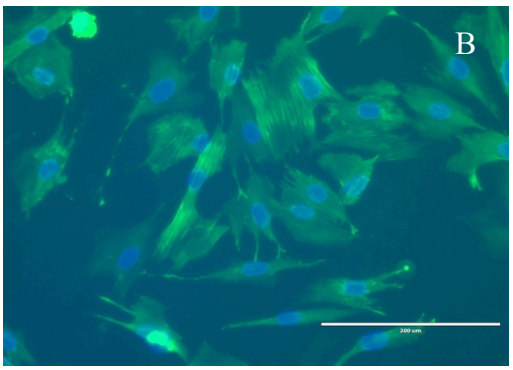
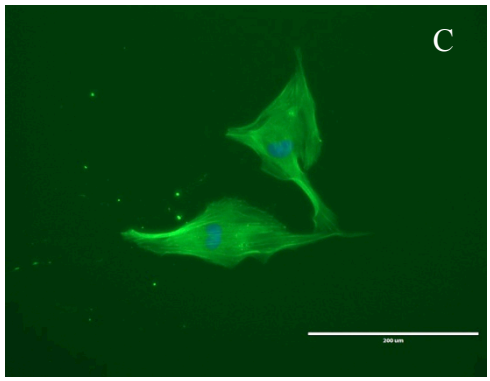
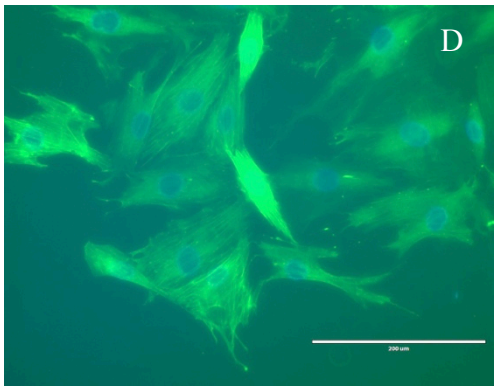
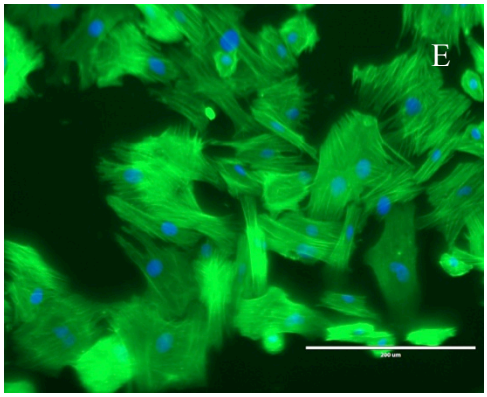
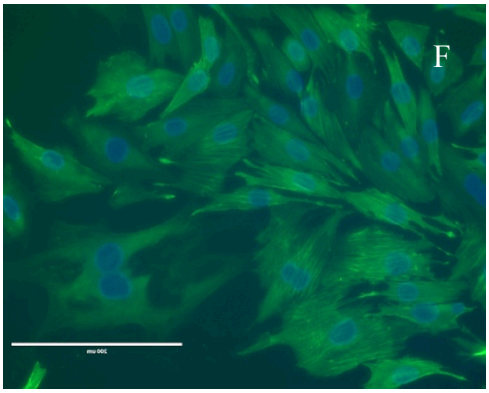
	Static Condition	Dynamic Condition
Control		
5nm AuNPs		
20nm AuNPs		

Table 4.1: The alpha-actin fluorescence images of the VSMCs with static and dynamic experimental conditions exposed to 5 nm & 20 nm citrate capped AuNP.

The VSMCs was stained to examine the alpha actin content to determine if a phenotypic shift occurred. By looking at the actin distribution and arrangement using immunofluorescence staining, these phenotypic changes can be visually observed. The VSMCs stained under the static conditions can be seen in **Figure 5.1.A**, **5.1.C**, and **5.1.E.** These VSMCs appear larger and shorter than the mechanical stretching/dynamic testing VSMCs, which indicates the synthetic phenotype expression. The cells under the mechanical stretching shown in **Figure 5.1.B**, **5.1.D**, and **5.1.F** appear more elongated and thinner, a significant indication of a contractile phenotype expression. Besides the shape and size of the VSMCs, the actin distributions of static and dynamic groups are also different. The group subjected to mechanical stretching has a very neat and parallel arrangement of the actin fibers within the cell, and this ordered arrangement supports contractile phenotype characteristics. The static condition cells do not show an ordered actin fiber orientation. The actin fibers appear to be randomly arranged, indicating that these cells might be expressing more synthetic phenotype characteristics.

Elastic Modulus from AFM Analysis:

The effects of the citrate capped AuNPs on the mechanical properties of the VSMCs can be seen from the elastic moduli results. The control cells cultured under static conditions had an elastic modulus of 7.63±1.61 kPa. When the VSMCs were treated with the 5 nm and 20 nm diameter citrate capped AuNPs, the elastic moduli of those cells were 5.72±1.46 kPa and 4.53±1.56 kPa, respectively. Statistical analysis of the elastic moduli of the static experimental group showed a statistical significance ($p<0.05$) indicating there is a big change between the control and the experimental group.

There was a significant statistical difference between the control cells and the cells treated with both the 5 nm and 20 nm AuNPs. The results showed that the nanoparticles had a softening effect on the mechanical properties of the cell. There was also a significant statistical difference between the VSMCs treated with the 5 nm versus the 20 nm AuNPs. This conclusion indicated that the increase in the size of the nanoparticles decreased the overall mechanical properties of the treated cells, at least for the static conditions. The cells treated with the two different size AuNPs showed different effects.

The controls cells subjected to mechanical stretching had an average elastic modulus around 11±1.10 kPa. The cells analyzed under mechanical stretching conditions were treated with 5 nm and 20 nm citrate capped AuNPs had elastic moduli of 8.26±1.15 kPa and 8.53±1.93 kPa, respectively. Statistical analysis showed there was only a significant statistical difference between the control cells and both nanoparticle treated group, but no significant difference between the 5 nm treated cells and the 20 nm treated cells. These results indicated that the nanoparticles caused a decrease in the mechanical properties of the VSMCs regardless of static or dynamic testing conditions, but the effect of particle size on the mechanical properties of the cells was only seen under static testing conditions. The elastic moduli of all the cells subjected to mechanical stretching were all significantly higher compared to the cells under the static experimental conditions.

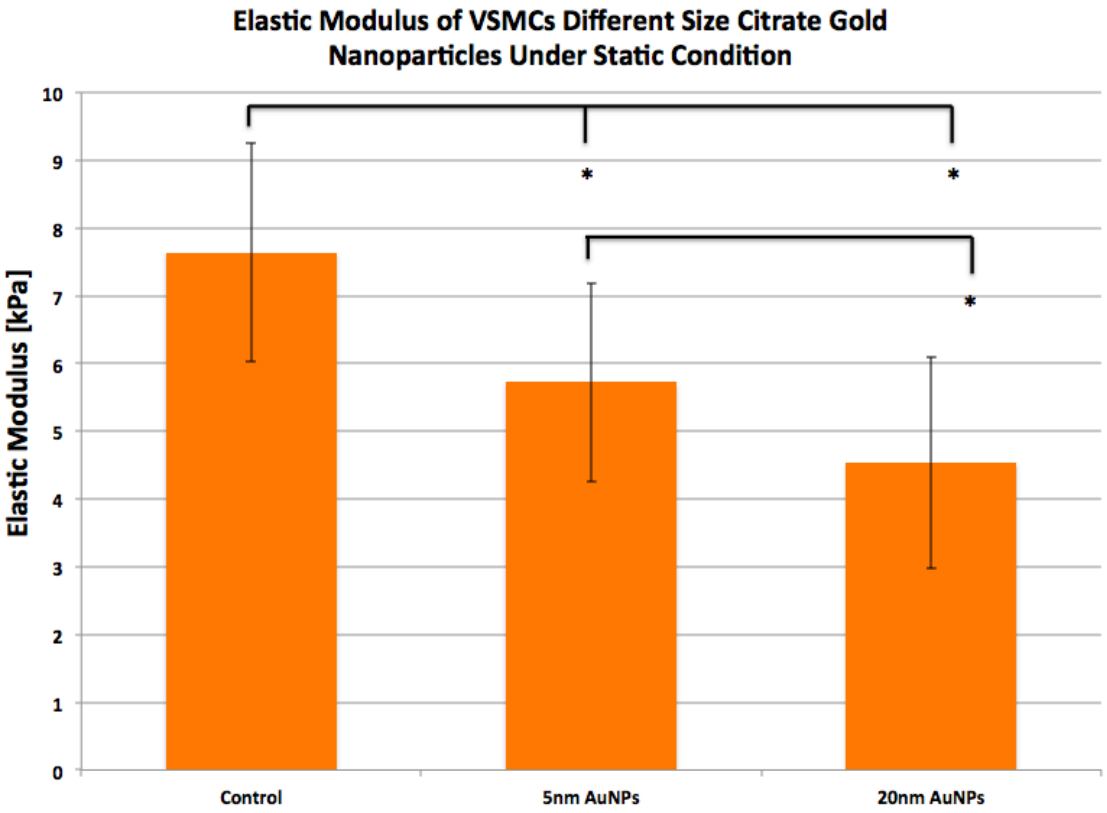


Figure 4.5: The elastic modulus of the VSMCs under static condition. The data showed there is a significant difference between the control and the nanoparticle treated cells as well as directly between 5nm AuNP treated and the 20nm AuNP treated cells.

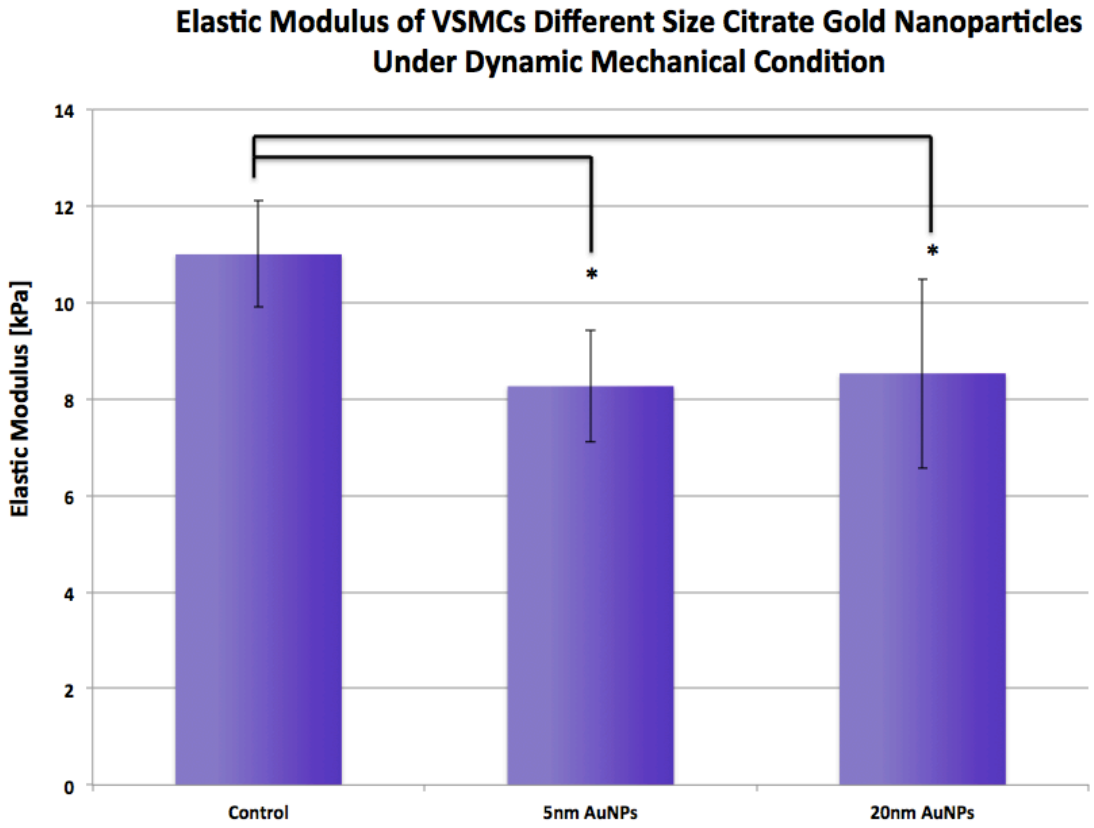


Figure 4.6: The elastic modulus of the VSMCs subjected to mechanical stretching condition. The data showed there is a significant difference between the control and the nanoparticle treated cells. There is not a statistical difference the two nanoparticle treated groups.

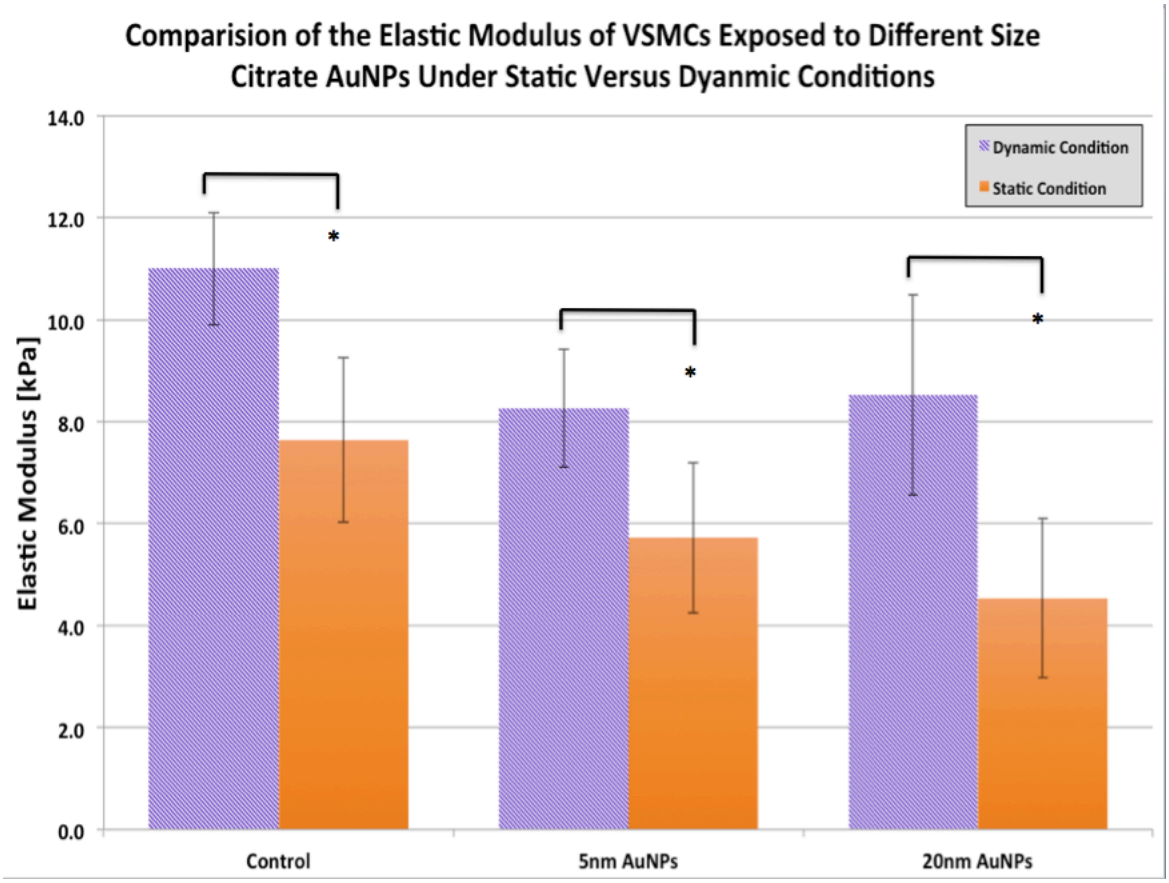


Figure 4.7: Comparison of the elastic modulus from the static experimental groups to the dynamic experiment groups. The data showed that there is a significant difference between the dynamic cell groups and the static cell groups. The cells subjected to mechanical stretching had a higher elastic modulus.

4.4 Discussion:

Based on the experimental results in this study, the 5 nm and 20 nm diameter citrate capped AuNPs do not appear to have any cytotoxic effects on the VSMCs. Both the LDH and MTT cytotoxic results in this study are similar to other literature findings. However, even though the particles are not cytotoxic to the cells, they show some influence on the mechanical properties of the VSMCs. Significant changes in the elastic moduli seen from the AFM results as well as the shift in morphology and alpha actin arrangement observed in the immunofluorescence staining, indicate that the AuNPs are decreasing mechanical properties and altering some of the characteristics of the VSMCs. This is a good indication of a phenotypic shift from a more contractile to a more synthetic expression.

The elastic moduli of the static versus dynamic groups showed that the VSMCs are more influenced by the nanoparticles under static conditions compared to dynamic stretching conditions. The difference between the control cells under static condition and the AuNPs treated groups indicated that the nanoparticles decreased the mechanical properties of the cells. The differences between the average elastic moduli of the 5 nm AuNP treated cells and the 20 nm AuNPs treated cells, suggested that the VSMCs exposed to larger particles have a slightly lower modulus of elasticity. The actin distribution and arrangement between the control and the static experimental groups also indicated a more synthetic phenotype due to the rounder cells and lack of orientation of the alpha-actin fibers. See **Figures 5.1.C** and **Figure 5.1.E**.

The cells subjected to mechanical stretching yielded similar results with a few key differences. The elastic moduli of the dynamic control VSMCs group was significantly higher compared to both the AuNPs groups, but the change in the moduli based on nanoparticle size was not observed. However, the effect on shape and size of the cells are still seen. The VSMCs treated with AuNPs are apparently larger and rounder compared to the control group, which has more elongated cells. The actin distribution and arrangement appeared similar between all the dynamic experimental groups. The actin fiber seems to orient in a distinct direction. This could be due to the presence of mechanical stimulation that can cause the ordering of the actin stress fiber in a certain direction.

Regarding the presence of mechanical stimulation, the AuNPs seems to be influencing the mechanical properties and overall morphology of the VSMCs. This observation could indicate a potentially adverse effect if these nanoparticles change the VSMCs enough to alter the main cell function. Another complication also arises because VSMCs play a critical role in the development and progression of atherosclerosis, and any alteration to the VSMCs could exacerbate or decrease atherosclerosis development. More testing needs to be performed to better understand the extent that the nanoparticles are affecting the cell morphology, mechanical properties, and overall function, and the ramifications these alterations have for cardiovascular diseases. Knowing that the AuNPs are affecting the VSMCs furthers the understanding of the interactions between the nanoparticles and cells but also presents other challenges for scientists and researchers to address further.

4.5 Conclusion:

Vascular smooth muscle cells exposed to citrate capped AuNPs cause changes in the cell morphology and decreased the mechanical properties of the cells, with or without mechanical stimulation. The size of the AuNPs only influences the mechanical properties of VSMCs when the cells are under static conditions. The nanoparticles are altering the alpha-actin formation within the cells. Future research needs to investigate the intercellular processes and activities to further understand how the nanoparticles are ultimately influencing the morphological changes and mechanical properties.

CHAPTER FIVE

RECOMMENDATION FOR FUTURE WORKS

The results in this study provide further insight into the world of nanoparticles and how they affect and interact with VSMCs. This project could be improved upon by performing polymerase chain reaction (PCR) or Western Blotting targeting specific markers to gather more evidence of phenotypic change due to the exposure of particles. This study could also be repeated with a larger range of particle sizes, such as 2 nm, 15 nm, 30 nm, and 50 nm, to further confirm and understand the effect of particle size on the VSMCs.

One of the main questions instigated by this study was where are these nanoparticles depositing once they are inside the cell? Some literary papers have reported that the nanoparticles are trapped in vesicles within the cells and others suggested the nanoparticle are able to interact freely with substances in the cytoplasm. Transmission electron microscopy or dual-photon microscopy techniques could be performed to analyze the ultimate location of nanoparticles within the cells. Understanding the nanoparticles location within the cell could provide a better comprehension and prediction of possible effects these particles could be having on the cell.

Another direction to take this research is to investigate how the exposure of these nanoparticles affects a co-culture of vascular endothelial cells (VECs) and vascular smooth muscle cells (VSMCs) cultured together. The novelty of this research is that the

investigation will utilize two cell lines compared to just one cell type, and this will simulate a more *in vivo* like environment. The study would look at the not only the nanoparticles and the VSMCs interaction, but also the interaction between the nanoparticles and the VECs as well as the VECs and the VSMCs. The results would provide a greater insight into the nanoparticles effects on the blood vessels *in vivo* using an *in vitro* model.

APPENDICES

Appendix A

Isolating Vascular Smooth Muscle Cells

- Sacrifice rat using CO_2 asphyxiation and cervical dislocation
- Remove aorta from subclavian origin to the diaphragm bifurcation and place in DMEM with pen/strep
- Use tissue culture hood to remove all adventitia and connective tissue. All adventitia must be removed to ensure you only attain VSMCs
- Cut vessel longitudinally so it lays flat and scrape off endothelium gently with scalpel blade and rinse thoroughly in DMEM with pen/strep
- Cut the vessel into ~5 mm squares and place them in digestion solution that includes 5 ml DMEM, pen/strep (1%), Collagenase type II (final concentration 175U/ml), and 10% FBS for 20 minutes
- Centrifuge at 500 rpm for 1 minute and remove the supernatant and wash the pellet with DMEM containing pen/strep
- Resuspend the pieces in another digestion solution containing DMEM, pen/strep (1%) Collagenase type II (final concentration 175U/ml), Elastase type III (final concentration 0.25 mg/ml) and 10% FBS for 1 hour. Be sure to shake the tube gently every 10 minutes until the vessel is gossamer thin. When the vessels are dissolved stop the digestion by diluting with equal volume of DMEM with pen/strep and 20% FBS
- Centrifuge 1000 rpm for 5 minutes and remove the supernatant

- Suspend cells in DMEM with pen/strep and 20% FBS and place in T-25 flask and put in incubator (37C, 5% CO_2). Don't change media for 72 hours as cells need sufficient time to adhere to flask.
- Maintain the cells using DMEM with 20% FBS and pen/strep until cells reach P4 then use 10% FBS

Appendix B

MATLAB Script for AFM Analysis

Hertz Model Analysis for Batch Export from MFP3-D AFM machine (reads text files generated by the computer)

```
function elasticity = massexcompile(folderin)
mainfolder = cd
format long
fnames = dir(folderin);
numfids = length(fnames);
cd(folderin);
%filtering out irrelevent "files" such as '.' and '..'
cellnames = {};
for c = 1:numfids;
    if 'C' == fnames(c).name(1) % 'C' represents the letter that the
relevent file names begin with
        cellnames{end+1} = fnames(c).name;
    end
end
%combine every 3 files and write
counter = 1;
numcell = length(cellnames);
numfile = 1;
elasticity = [];

% OMIT THIS WHILE LOOP IF YOU WANT TO LOAD CELLS INDIVIDUALLY
while counter <= numcell
    a = load(cellnames{counter});
    c = load(cellnames{counter+2});

    cd(mainfolder);
    elasticity(end+1,[1,2]) = elast_analysis(c,a,mainfolder);
    counter = counter+3;
    cd(folderin);
    %numfile = numfile+1
end


function e = elast_analysis(c,a,mainfolder)
cd(mainfolder)
format long
k = 0.1416; %spring constant value N/m
v = 0.5; %poisson's ratio
R = 2.5*10^-6;  % tip radius in meters
L = 10*10^-9 ;     %lower bound for elasticity (in m from contact
point)
U = 700*10^-9 ;   %upper bound for elasticity (in m from contact point)
```

```
%adjust deflection
ak = a.*k;
%filter deflection values
d = AFM_butter(ak);
%Separation of extension and retraction
l = floor(length(c)/2);
xe = c(200:l);% add 200 in order to omit first several data points
(irratic behavior due to filtering
ye = d(200:l);
if rem(length(c),2)==0;
    xr = c(end-200:-1:l+1); % subtract 200 in order to omit first
several data points
    yr = d(end-200:-1:l+1);
    else
    xr = c(end-200:-1:l+2);
    yr = d(end-200:-1:l+2);
end

%correct x,y offsets
[xe,ye] = xycorrect(xe,ye);
[xr,yr] = xycorrect(xr,yr);

format long;
erange = [];
for i = [1:1:length(xe)];
    if xe(i)>=L && xe(i)<=U;
        erange(end+1) = i;
    end;
end;
rrange = [];
for i = [1:1:length(xr)];
    if xr(i)>=L && xr(i)<=U;
        rrange(end+1) = i;
    end;
end;

emodulus = mean((3.*ye(erange).*(1-
v^2))./(4.*xe(erange).^(3/2).*R.^(1/2)));
rmodulus = mean((3.*yr(rrange).*(1-
v^2))./(4.*xr(rrange).^(3/2).*R.^(1/2)));

e = [emodulus, rmodulus];

function [i] = AFM_butter(x) %applies butterworth filter to data
format long
[b,a]=butter(3,.025);
i=filter(b,a,x);
```

```
function [xc,yc] = xycorrect(x,y)
s = 0.002; %slope sensitivity


%correction for y
format long
region = [1:length(x)/4];
slope = polyfit(x(region),y(region),1);
yci = y-(slope(1).*y);

%correction for x
numslope = diff(yci)./diff(x);
index = 1;
condition = 0;
contactx = 0;
while condition == 0 && index ~= length(numslope)
    if numslope(index) > s && mean(numslope(index:5:index+200)) > s;
        condition = 1;
        contactx = index;
    end
    index = index+1;
end
xc = x-x(contactx);

% correct again for y
yc = yci-yci(contactx);
```

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